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2,4-D Hyper Accumulation Induced Cellular Responses of *Azolla pinnata* R. Br. to Sustain Herbicidal Stress

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Abstract: In the present experiment with ongoing concentration (0 μM, 100 μM, 250 μM, 500 μM and 1000 μM) of 2,4-D, the responses of *Azolla pinnata* R.Br. was evaluated based on cellular functions. Initially, plants were significantly tolerated up to 1000 μM of 2,4-D with its survival. This was accompanied by a steady decline of indole acetic acid (IAA) concentration in tissues with 78.8% over the control. Membrane bound H^+-ATPase activity was over expressed within a range of 1.14 to 1.25 folds with activator (KCl) and decreased within a range of 57.3 to 74.6% in response to inhibitor (Vanadate) application. With regards to IAA metabolism, plants recorded a linear increase with wall bound oxidase activity up to maximum concentration of 2,4-D. The variations were more moderated when wall bound IAA-oxidase recorded a linear increase proportionate to the 2,4-D concentrations. This was more extended with the presence of different isoforms of IAA-oxidase which was much more pronounced with distinct polymorphisms of expressed proteins, however, not independent to the 2,4-D concentrations. Polyamines like spermine, spermidine and putrescine (spm, spd and put) were not consistent in concentration with the dosages of 2,4-D. Besides these, plants were induced to apoplastic NAD(P)H oxidase activity maximally by 1.6 folds under 500 μM 2,4-D over control. Still, putrescine responded more or less consistently and recorded maximally 11.9 folds at 500 μM 2,4-D as compared to the control. NAD(P)H oxidase activity recorded maximally 1.6 folds against control and remain consistent throughout the concentrations of 2,4-D. GPX along with APX were more linear in responses through the concentration of 2,4-D except CAT as compared to control. On enzymatic antioxidative activity, peroxidases (GPX and APX) were overexpresed in a similar manner except for catalase with a non-significant rise. In stabilization of cellular redox, glutathione reductase attended maximum value by 2.45 folds at 1000 μM evidenced with significant variations in protein polymorphism. The sensitivity of 2,4-D also appeared in *Azolla* with a maximum loss of nucleic acids as documented by the comet assay. Moreover, the *Azolla* might have some DNA damage protective activity as evident using frond extract with plasmid nick assay. Therefore, *Azolla* plants with its cellular responses is evident to sustain against the 2,4-D herbicidal stress and may be granted in bio remediation process for the contaminated soil.

Keywords: *Azolla*; 2,4-D; antioxidative enzymes; comet assay; HPLC

1 Introduction

Plants respond in various ways to changing environment for its extremities or any sort of fluctuations. Man made environmental pollutants like xenobiotics has been alarming for agricultural pollution since long back. Discharge of herbicides belonging to such category has been important for their detrimental effects on soil through it's fertility as well as micro flora [1]. This is facilitated either through direct contamination or as residual effects of herbicides. With the advent of herbicidal exposure following its toxicity, few plant species have already exhibited their tolerant potentials through their various cellular and physiological responses. According categories of herbicides, mostly with pre and post-emergent in exposition, plant species, still, few in number recorded as resistant types. Systemic herbicides point out the possible cites of hindrances of cellular metabolism in biochemical paths [2]. Thus, absorption of herbicide residue following its translocation and finally targeting the metabolic fluxes would be the basis for differentiation between resistant and susceptible species [3]. Plant species may alter their activities of metabolism in an indirect way to down regulate with the increasing concentration of herbicides like glyphosate. With broad leaf species (like Bean), glyphosate down regulates the receptor sensitivity to gibberellic acid on the membrane [4]. Abscisic acid (ABA), a common growth retardant for membrane permeability is ensured with over production under round up (Glyphosate) application in soil [5]. Auxin and its related natural derivatives are markedly limited with their concentration to regulate cellular metabolism [6]. Therefore, membrane integrity and its transport would be other facets for sensitivity of herbicides like xenobiotics [7]. There are ample evidences for over-expression and thereby elicitation of few chemical residues towards the abiotic stresses tolerance. In few aquatic weeds a clear compatibility exists for salicylic acid and related phenolics accumulation to support the tolerance against organo-mercurales as industrial effluents. Therefore, membrane protein functions and related proteins would be important to justify the herbicide mediated toxicity in plants. In downstream regulation, plants always are to compromise with gene(s) regulation where DNA damage and impairment for transcription are the most crucial. Dislocation of nucleic acid bases, nicking of DNA strands are the initiation for impaired gene functioning [8]. From significant amount of works, herbicides are registered for genotoxicity through reactive oxygen mediated loss of bio-molecules [9]. Membrane lipids and proteins, other heterocyclic unsaturated residues, nucleic acids are the targets of such reactive oxygen species (ROS). Therefore, origin of ROS through cellular paths is expected to be regulatory in herbicide tolerance in few resistant species. Besides other compounds, in resistance to plant systems, polyamines have been implicated against different stressors. Polyamines though ubiquitous in nature are still discriminatory in functioning according to genotypic perception of stress signals and its downstream reactions. Polyamines mostly have been in practice for stress amelioration in higher plants but still not to be significant in lower groups. Therefore, any such plant groups with moderate to higher herbicidal sensitivity are expected to employ polyamines to interact with toxic chemicals.

Few weeds are often displayed with wider spectrum of tolerance to various xenobiotic residues, herbicides being the least mentioned therein [10]. Aquatic weed species including few pteridophytic taxons are well ahead of hyperaccumulation of heavy metals and metalloids, organo-mercurales, poly-chlorinated hydrocarbons, synthetic Azo dyes etc. All these behaviours undoubtedly establish those species as better hyperaccumulator of toxic residues of those chemicals. With this background we have selected *Azolla pinnata* R.Br., an aquatic fern with their better adaptability under waste water containing metals as well as herbicides [11]. From various corner of cellular responses *Azolla* documented two major domains of reactivity: first, sequestering of the toxic substances in non-cellular spaces and second, minimize the ROS-induced oxidative damages. In addition, a burst of ROS in *Azolla* could elicit some reactions through over expresses few genes 2,4-Dichlorophenoxy acetic acid (2,4-D), a common pre-emergent herbicide. In comparison to sensitive species *Azolla* established to be well hyper-accumulator when cultured in fields with residual toxicity [12]. With more insights for such a regulation of 2,4-D

effects in plant, *Azolla* are undoubtedly established to be a bio-control measure for herbicidal toxicity [13]. Therefore, more insights would be in pursuit of better option of phyto remediation with this fern species in herbicidal contaminated soil and realize the active principle(s) for such tolerance. Moreover, the contributing physiological/cellular responses would serve as selection criteria for other related weed species from natural populations. With this view, we conducted the experiment, where *Azolla* exhibited a wider range of tolerance to simulated 2,4-D concentration. We analyzed and discussed the significant cellular reactions from membrane functions to nuclear responses in contiguous to 2,4-D stress and probable tolerance to 2,4-D in this species.

2 Materials and Methods

2.1 Experimental Plant Material

The present *in vitro* experiment was conducted in the Plant Physiology and Plant Molecular Biology Research Laboratory, Department of Botany, University of Kalyani West Bengal, India. The plants (*Azolla pinnata* R. Br.) were collected from the aquatic ponds in the University premises and were grown in cemented tank containing fresh water for 7 days and thereafter, the plants were incubated with ¼th Murashige and Skoog's Medium in the laboratory condition for 3 days for acclimatization followed by varying concentrations (0 µM, 100 µM, 250 µM, 500 µM and 1000 µM) of 2,4-Dichloro-phenoxy acetic acid. The treated plants were kept under ambient condition of 36 ± 1°C of temperature, 70–80% of RH and photoperiod of 13–11 h light and dark photoperiod for 7 days. After 7 days, these treated plants were stored at −80°C for further experimental purpose.

2.2 Experimental Methods

2.2.1 Determination of IAA-Content Through HPLC

Both the control and treated plant samples were analysed with a high performance liquid chromatography (HPLC) system, equipped with ultraviolet (UV) detector absorbing at 250 nm and a C18 column (Agilent, USA). The extraction and purification of plant samples were done according to Dobrev et al. [14] with slight modification. The mobile phases used were methanol: water (80:20) at a flow rate of 1.0 ml/min. The sample injection volume was 10 µl and retention times for the respective peaks were compared to those of internal standard. Quantification was done by comparison of peak area [15].

2.2.2 Estimation of Plasma Membrane Bound H^+-ATPase Activity (EC 3.6.3.6)

Determination of plasma membrane bound H^+-ATPase activity was done taking 2.0 g fresh roots from control and treated samples and homogenised in liquid nitrogen with an extraction buffer containing 250 mM sucrose, 10 mM EDTA, 3 Mm PMSF, 150 mM KCl, 10 µl protease inhibitor cocktail, 0.1% BSA, 2% PVP, 1 mM DTT and 25 mM HEPES-KOH (pH 7.8). The homogenate was centrifuged at 13,000×g for 25 min at 4°C followed by ultra centrifugation at 80,000×g for 1 h and the pellet was recovered and resuspended in the suspension buffer containing 200 mM sucrose, 5 mM EDTA, 2 mM DTT, 1 mM KCl, 50 mM HEPES-KOH (pH 7.8). Finally, the H^+-ATPase activity was estimated from the recovered pellet according to Janicka-Russak et al. [16] with an assay mixture containing 25 mM Tris-MES (pH 7.0), 5 mM ATP.

2.2.3 Determination of IAA-Oxidase Activity

IAA-oxidase activity was determined according to Ebrahimzadeh et al. [17] with slight modifications. 0.5 g of fresh roots was washed thoroughly with de-ionized water, blotted dry and homogenized with 0.1 M phosphate buffer (pH 7.0). Then the homogenate was centrifuged at 15,000×g for 20 min at 4°C. The supernatant was used as an enzyme source. Thereafter, the supernatant was collected and added within assay mixture containing 3 ml 0.1 M phosphate buffer (pH 6.5), 2 ml para-coumaric acid (0.5 mg ml^{-1}), 0.5 ml 0.002 M $MnCl_2$ and 4 ml IAA (0.05 mg ml^{-1}) was added to start the reaction. Thereafter, the reaction mixture was incubated in dark within shaker at 30°C. The enzymatic reaction was started by

addition of 2 ml of assay mixture. After 1 h of incubation 5 ml of sulphuric acid-ferric chloride ($FeCl_3$) mixture (1:5) was added to it and mixed well and kept in dark for 30 min. The absorbance was recorded at 535 nm.

For *in-gel* analysis of IAA-oxidase, 10% native polyacrylamide gel electrophoresis (PAGE) was used at 10 V/lane under 4°C. The detection of specific polypeptide band was resolved in 250 mM phosphate buffer (pH 7.0) and stained with o-dianisidine solution (20 mg o-dianisidine in 1 ml of warm glacial acetic acid) followed by addition of 500 µl of 0.5% H_2O_2 with gentle agitation.

2.2.4 High Performance Liquid Chromatography Analysis of Polyamine

Polyamine was extracted from 1.0 g lyophilized leaves by homogenizing it in 5% (v/v) perchloric acid at 4°C. The homogenate was centrifuged at 15,000×g for 15 min and the supernatant stored at −20°C. The dansylation was done by adding 1.5 ml of carbonate buffer (pH 9.0) to 1 ml of supernatant, followed by the addition of 1 ml of 5 mg/ml dansyl chloride (dissolved in acetone). After 1 h of incubation at 50°C in the dark, 100 µl Proline (100 mg ml^{-1}) was added to stop the reaction and the reaction mixture was dissolved with 3 ml of n-heptane [18]. Then the organic phase was evaporated and re-suspended in 1.5 ml acetonitrile, and the solution was used for HPLC analysis using a reverse phase C18 column (Agilent, USA). The flow rate was 1.0 ml/min and the detected through UV-detector at 250 nm wavelength. The separation was done on a solute involving HPLC grade water (A) and 100% acetonitrile (B): 20% A, 80% B, 10 min.

2.2.5 NAD(P)H-Oxidase (NOX) (EC 1.6.3.1) Activity Assay

500 mg of fresh plant tissues were homogenized with Liquid nitrogen using extraction buffer containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.2), 1 mM ethylene diamine tetra acetic acid (EDTA), and 2.5 mM dithiothreitol (DTT) and centrifuged at 10,000×g for 15 min at 4°C. The supernatant was the extracted membranes and the membrane fraction was separated from the supernatant by again centrifugation at 80,000×g for 30 min, according to Janeczko et al. [19]. Then the pellet was resuspended in a Tris–HCl (pH 7.2) and used for further analysis. The resuspended solution was mixed with an assay mixture containing 10 mM Tris–HCl (pH 7.5), 1 mM sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT), 1 mM nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), and 100 µl of membrane proteins. The reduction of XTT by O_2^- was recorded at 492 nm according to Able et al. [20]. The O_2^- generation rate was calculated using an extinction coefficient of 2.16 × 104 M^{-1} cm^{-1}.

The native polyacrylamide gel analysis was done at 70 V at 4°C according to Laemmli [21]. NADPH-dependent superoxide producing capabilities of the proteins were assayed by the blue formazon (NBT) reduction method according to Sagi and Fluhr [22] with some modifications. The gels were incubated in the dark for 20 min in a staining solution containing 50 mM Tris–HCl (pH 7.5), 0.2 mM NBT, 0.1 mM $MgCl_2$, and 1 mM $CaCl_2$. NADPH (0.2 mM) was added and the reaction was terminated by the immersion of gels in distilled water.

2.2.6 Determination of Different Antioxidative Enzyme Assay

Preparation of crude enzyme extract from 0.5 g of fresh plant samples were crushed in liquid nitrogen and homogenized with 100 mM phosphate buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mM PMSF, 100 mM EDTA, 10 mM DTT and 2% PVP. The homogenate was centrifuged at 15,000×g for 20 min at 4°C. The supernatant was kept for further *in-vitro* and *in-gel* analysis. For *in-gel* analysis of different their different isoforms, Guaiacol peroxidase (GPX), Ascorbate peroxidase (APX), Catalase (CAT) and Glutathione Reductase (GR) were separated in non-denaturing polyacrylamide gels according to Laemmli [21]. Equal amounts of protein extracts (25 mg) were loaded on 8% (CAT) or 10% (GPX, APX, GR and CAT) polyacrylamide gels.

2.2.6.1 Guaiacol Peroxidase (GPX) (EC 1.11.1.7) Activity Assay

Extraction of enzyme from leaf sample was done by complete homogenization under liquid nitrogen in cold room. 50 mg of lyophilised sample was extracted in a buffer containing 100 mM Tris-Cl (pH 7.8) buffer containing 1 mM PMSF, 10 mM $MgCl_2$, 100 mM EDTA, 10 mM DTT and 2% PVP, 0.1% protease inhibitor. The homogenate was separated into supernatant with centrifugation at 15,000×g for 20 min at 4°C. The soup was taken for crude enzyme source and kept at −20°C for further use. For activity of GPX, the 100 µg equivalent protein of the supernatant was incubated in an assay buffer containing 50 mM Potassium phosphate buffer, 0.5 mM ortho-dianisidine and 0.5% H_2O_2. The changes of absorbance were read at spectrophotometer with 15 min duration and activity was calculated from Δt with extinction coefficient of H_2O_2 (0.485) as described Ammar et al. [23].

For *in-gel* analysis of GPX, gel was incubated within a staining solution containing 50 mM sodium phosphate buffer (pH 6.5), 0.5 mg/ml o-dianisidine (dissolved in acetic acid) and at last 3 mM H_2O_2 with gentle agitation in dark.

2.2.6.2 Ascorbate Peroxidase (APX) (EC 1.11.1.11) Activity Assay

For APX, the activity was monitored in a reaction mixture containing 100 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate and 0.2 mM H_2O_2. The enzyme protein was added with 100 µg equivalent supernatant from the leaf sample. The activity was calculated from changes in absorbance with 290 nm as suggested by Davletova et al. [24].

For *in-gel* analysis of APX, the gels were incubated within an incubation mixture containing 50 mM sodium phosphate buffer (pH 7.5) and 2 mM ascorbate for 30 min, followed by incubation in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 15 min. Thereafter, the gel was stained with 50 mM sodium phosphate buffer (pH 7.5) and 2.45 mM nitro blue tetrazolium for 15 min with gentle agitation and the reaction was terminated by adding 28 mM TEMED [25].

2.2.6.3 Catalase (CAT) (EC 1.11.1.6) Activity Assay

CAT activity was determined according to Velikova et al. [26]. The supernatant was incubated in an assay mixture containing 100 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The activity was determined by recording the absorbance at 240 nm and activity was determined using the extinction coefficient of H_2O_2 0.036 mM^{-1} cm^{-1}.

For *in-gel* analysis of CAT, gels were stained according to Shah et al. [27] with slight modifications. The gels were incubated in 3.25 mM H_2O_2 for 20 min and the bands were resolved in a staining solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride for 5 min and then fixed with 1% HCl.

2.2.6.4 Glutathione Reductase (GR) (EC 1.8.18.7) Activity Assay

GR activity was determined by the glutathione dependent oxidation of NAD(P)H according to Cakmak et al. [28]. The enzyme was extracted using 250 mM phosphate buffer (pH 7.5), 100 mM EDTA, 2 mM PMSF, 10 mM $MgCl_2$, 10 mM β-ME and 5% PVP. The assay mixture containing 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 2 mM NAD(P)H, 30 mM oxidized glutathione. The reaction was started by adding 200 µl enzyme extract and the gradual decrease in absorbance was recorded at 340 nm for 1 min. The activity was calculated using molar extinction coefficient of NAD(P)H (6.22 $mM^{-1}cm^{-1}$) and expressed as mM NAD(P)H oxidized $min^{-1}mg^{-1}$ of protein.

For *in-gel* analysis of GR, the gels are incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 0.7 mM 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH for 1 h in dark. The reaction was terminated by adding 7.5% (v/v) glacial acetic acid to the staining buffer solution [25].

2.2.7 Determination of Nuclear Disintegration by Comet Assay

The nuclear disintegration study was done by alkaline comet assay according to Tice et al. [29]. The nuclei were isolated from *Azolla pinnata* R.Br. leaves and roots; then for analysis, the low melting agarose plated frosted slides were placed within the alkaline electrophoresis buffer (350 mM NaOH and 1 mM EDTA; pH > 12) at 4°C for 30 min at 25 V. The slides were stained within EtBr (20 μg ml^{-1}) solution. Thereafter, the data were recorded using image analyser (Kinetic imaging; Andor Technology, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1) and image-analysis software (Komet 5.5). The results were represented as comet tail DNA (%).

2.2.8 Plasmid Nick Assay

Plasmid DNA nick assay was done according to Lee et al. [30]. Fenton's reagent was used for generation of hydroxyl free radicals (OH^-) which reacts with pUC19 plasmid DNA causing DNA damage. Methanolic leaf extract of *Azolla* (20 μg/ml) were added to the reaction mixture (20 μl) containing 0.5 μg of pUC19 and Fenton reagent (30 mM H_2O_2, 50 μM ascorbic acid, 80 μM $FeCl_3$). After 30 min of incubation at 37°C the analysis was done by running the samples on 1% agarose gel electrophoresis (Mini Sub-Cell, Bio-Rad) followed by ethidium bromide staining. Gel documentation system was used to visualize the bands.

2.2.9 Statistical Analysis

The experimental data represented as mean of three replicates (n = 3) ± SE. Each bar showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$. Microsoft Excel 2007 software was employed for computation and data analysis.

3 Results

3.1 Variations of IAA Metabolism Through Its Content, Oxidation and Trend in H+-ATPase Activity

The present work is depicted with some significant observations where *Azolla* exhibited different cellular and molecular responses against 2,4-D treatments [31]. In addition to the tolerance of 2,4-D, it established a significant change in IAA levels (Fig. 1).

This undoubtedly establishes the encroachment of 2,4-D on IAA concentration to the cellular metabolism. On account of IAA catabolism, the present study with *Azolla* exhibited interesting phenomenon where IAA-oxidase varied significantly ($p \leq 0.05$) according to herbicide concentrations (Fig. 2a). The results typically exhibited over-expression of IAA-oxidase activity which ranges from 2.3 to 6.8 μM IAA oxidised mg^{-1} min^{-1}. On comparative basis, the oxidase activity had a sharp increase on a linear trend and maximally induced under 1000 μM of 2,4-D and scored 6.18 folds increase over the control in a dose dependent manner of 2,4-D. It was more significant to note that the IAA-oxidase activity under 2,4-D treatments had an inverse relationship to the cellular IAA concentration. The expression of IAA-oxidase activity had variation with its polymorphism, exhibiting typical isozymic patterns under native PAGE (Fig. 2b). Thus, two distinct polypeptide bands scored varying intensities of expressed proteins with 2,4-D concentration. The bands of I and II had significantly minimized in intensities under 2,4D as compared to control, still, band I regardless of concentration of IAA had better resolution than other assuming more contributory in IAA oxidation.

IAA showed significant association with membrane bound protein and their activities, particularly for those of ion-transporter rendering the cell wall extension. H^+-ATPase would be one of those studied in the present experiment under varying concentration of 2,4-D. Under varying concentration of 2,4-D the partial purified membrane proteins of H^+-ATPase of leaf was assayed in presence of both activator as well as inhibitor (Fig. 3).When the protein was incubated in assay mixture, a steeper rise in activity was recorded under activator as KCl. On the contrary, inhibitor as vanadate significantly curtailed the activity in a proportionate to 2,4-D through increasing 2,4-D doses. Thus, maximum up regulation recorded as

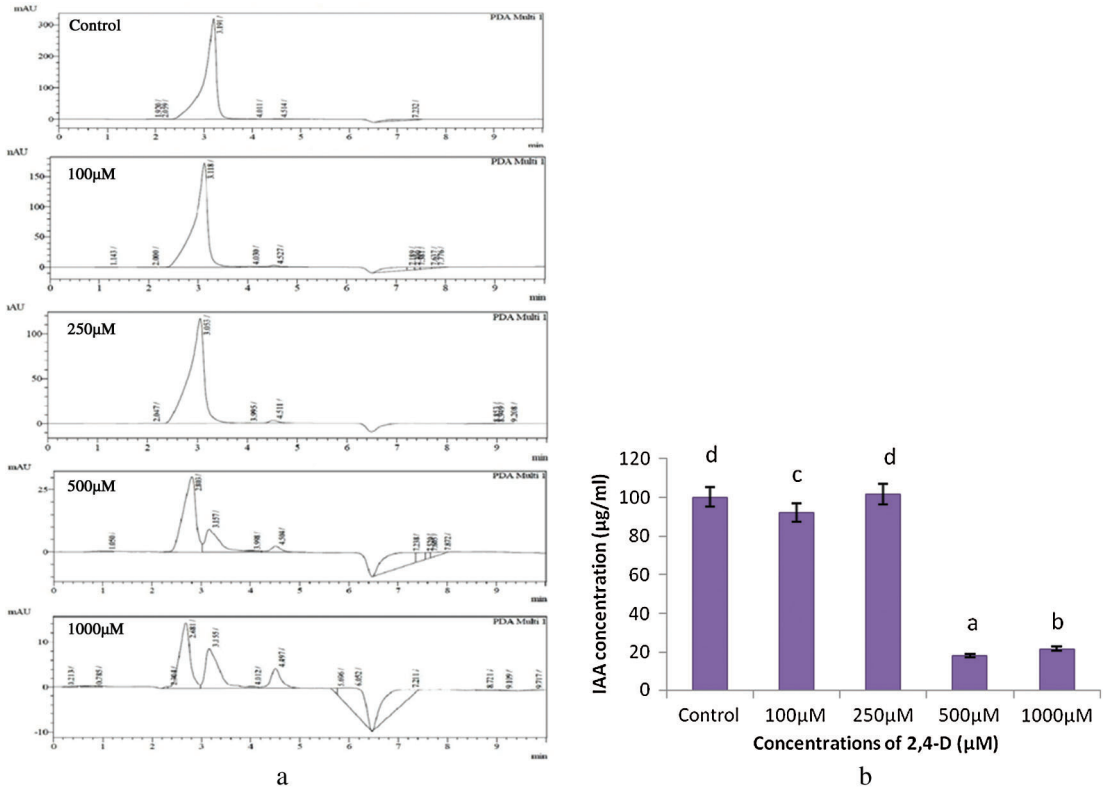

Figure 1: Determination of IAA-content through chromatogram (a) of high performance liquid chromatography and quantification (b) from peak intensities. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

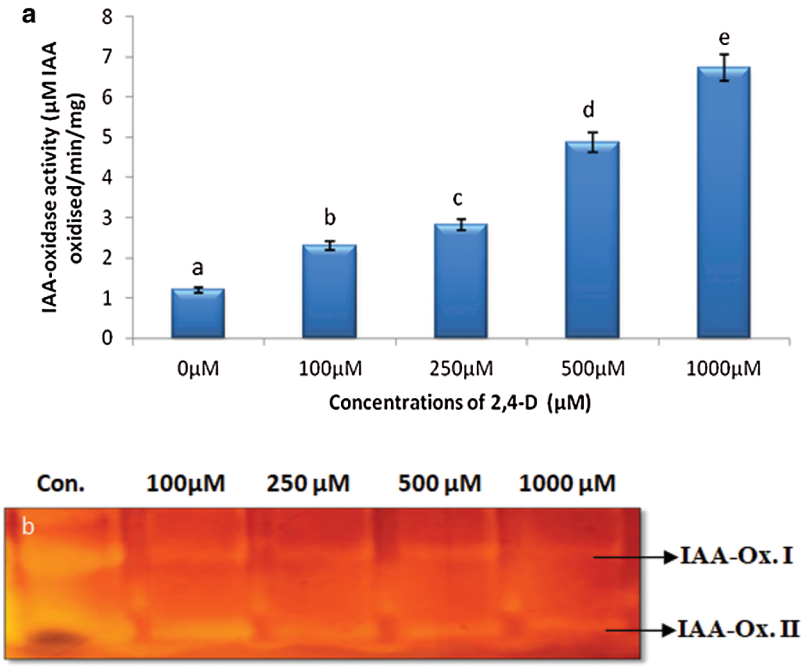

Figure 2: *In-vitro* (a) and *in-gel* (b) activity of IAA-oxidase activity (µM IAA oxidised mg^{-1} min^{-1}) under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

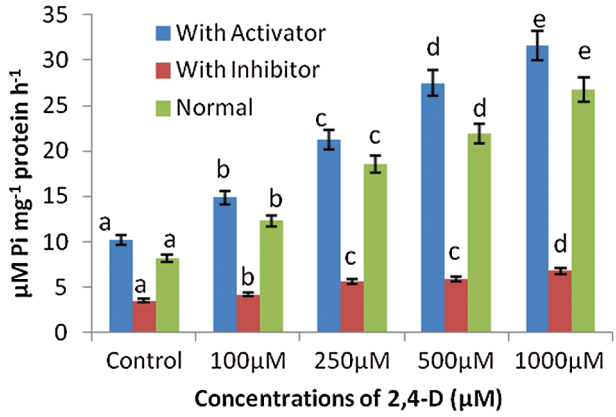

Figure 3: *In-vitro* activity of H^+-ATPase activity (µM Pi mg^{-1} protein h^{-1}) under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

compared to normal (without activator/inhibitor) was 1.17 folds for activator. The H^+-ATPase activity under inhibitor treatment was sensitive and showed downregulation by 78.48% as compared to activator treatment under same 2,4-D concentration. Still, when H^+-ATPase activity was monitored under normal condition a linear rise ranging from 1.5 to 3. 26 folds was featured through the 2,4-D concentration. Therefore, ATP hydrolysis through simulated pH gradient by acquisition of H^+ on membrane may be crucial for 2,4-D toxicity in this aquatic fern species.

3.2 NAD(P)H-Oxidase Activity, Polymorphism and Free Polyamine Concentration

Under the varying 2,4-D treatments (0–1000 µM) in simulated condition, *Azolla* recorded a severe change in cellular redox. This was monitored with *in-vitro* analysis of membrane bound NAD(P)H-oxidase (NOX) activity and *in-gel* studies when separated through non-denaturing PAGE. Plants responded to develop an antioxidative burst as monitored through NOX activity (Fig. 4a). This showed an almost linear relationship with up regulation in a dose dependent manner. The variation was significant at $p \leq 0.05$, where the activity was maximized by 1.6 folds from the plants under 1000 µM of 2,4-D treatments. Still, the intermediate concentrations of the treatments were recorded no significant variation among those. The cell membrane bound protein with NOX activity was recovered from plants and partial purification following separation through electrophoresis system demarked the possible isoforms. Fig. 4b documented that plants were over expressed with significant variations of NOX activity induced by 2,4-D against control. As a function of 2,4-D concentration, a single polymorphic band resolved which had an inconsistent expression patterns as revealed from band intensities through varying 2,4-D concentration. From densitometry analysis, the maximum expression was recorded at 500 µM of 2,4-D concentration which was subdued beyond this concentration of 2,4-D. This may be a possible indication of the tolerance level of herbicide that regulates the expression potentials of ROS through NOX activity in *Azolla* as presented herein. The presence of NOX protein was confirmed with absence of any polymorphic bands in lane of the gel run heat killed enzyme or BSA protein as internal control.

Plants after recovering from 2,4-D concentration were extracted with free polyamine determining the contribution for tolerance to herbicides. The plants recorded quite inconsistent trend for each polyamine fractions (spm, spd and put) under increasing concentration of 2,4D. Initially, from the observation of HPLC chromatogram using peak area and height against standard concentrations of polyamine, it recorded no significant variation for spermine, spermidine, and putrescine concentration under control condition (Fig. 5). Still, on total polyamine basis, 1000 µM 2,4-D experienced the maximum values by

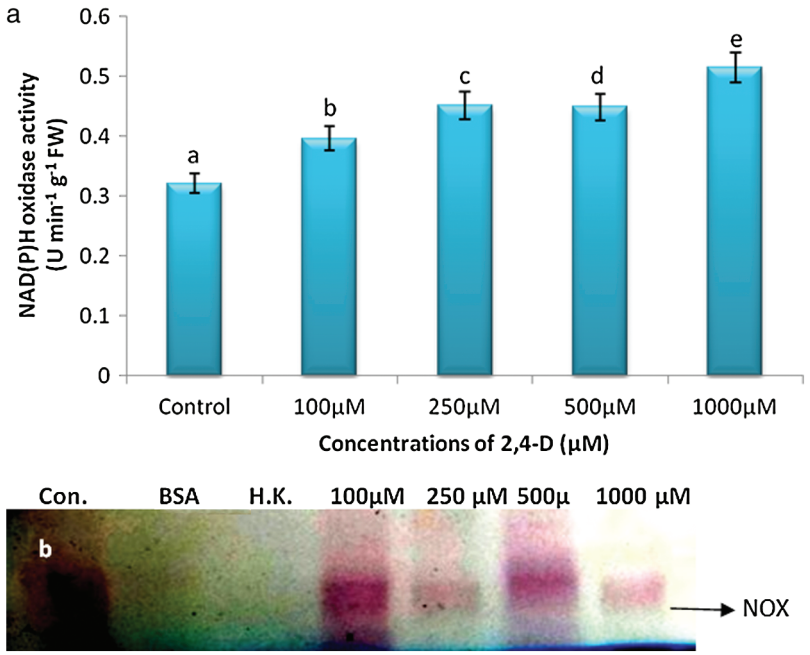

Figure 4: *In-vitro* (a) and *in-gel* (b) activity of NAD(P)H-oxidase activity (µM Pi mg^{-1} protein h^{-1}) under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

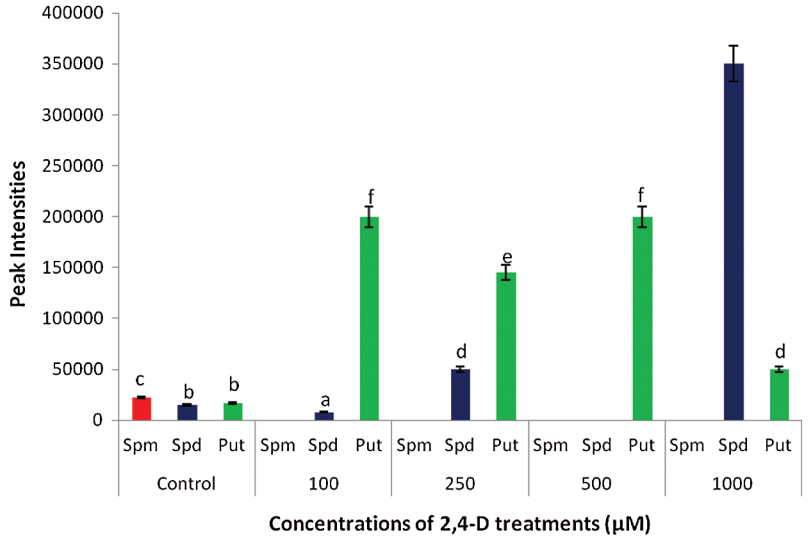

Figure 5: Determination of polyamine through high performance liquid chromatography and quantification from peak intensities. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$ (Spm: spermine, Spd: spermidine, Put: putrescine)

7.36 folds compared to control. Interestingly out of polyamine fractions spm were not detected in any of the ongoing concentrations of 2,4-D and thereby its depletion would be a identifying trait for 2,4-D concentration that recorded maximally at 500 µM. Likewise, spd had its peak value only at 1000 µM of 2,4-D concentration which exceeded 23.33 folds in content than control.

3.3 DNA Damage and Its Amelioration by Antioxidative Paths

From the present study the *Azolla* plants might be expected to have some cellular damages under 2,4-D mediated ROS over accumulation. This was documented by lysis of nucleic acid, however, DNA through comet assay (Fig. 6a). Impact of herbicides has a direct relationship to release the DNA material from lysis of nuclear membrane in the form of comet. So, it may be speculated that herbicide may act as detrimental to lose the nucleic acid as recorded with a significant increase in comet tail Fig. 6b represented the variation in comet tail length which had significantly ($p \leq 0.05$) varied by 31.6 to 37.9 folds in last two maximum concentrations of 2,4-D over control.

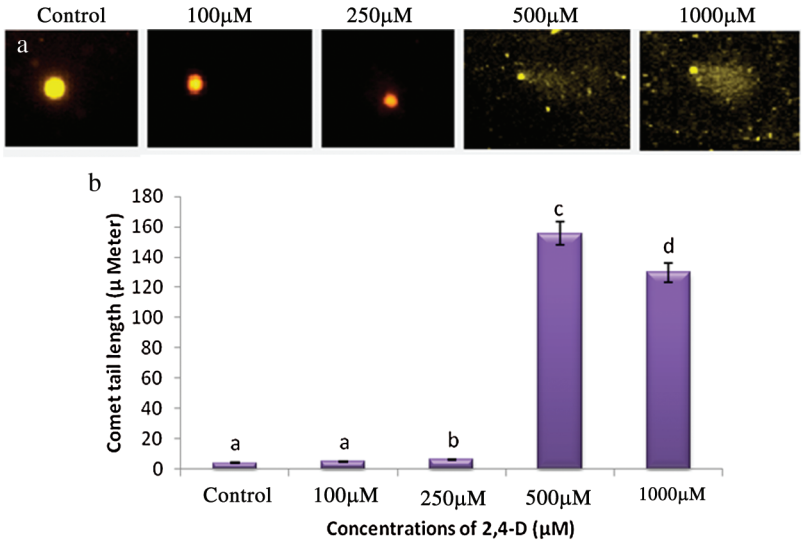

Figure 6: Images of comet plates (a) and graphical (b) representation of comet tail length under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

In another assay the methanolic extract of *Azolla* plants was tested to monitor the potential of organic residues to reverse the DNA damages. Fig. 7 represent the plasmid DNA nick assay where different sets constituting incubations of leaf extract with plasmid in presence and absence of Fenton's reagent. The panel showed the compatible nature of plasmid DNA denaturing under 2,4-D and Fenton's reagent respectively in Lane-4 and 3. A single band in those lanes may be evident for ROS induced oxidation of nucleic acid from native configurations of plasmid DNA. On the contrary lane-1 which represents the native plasmid had its distinct appearance of super coiled band with maximum concentrations of DNA. *Azolla* extract in incubation mixture of plasmid DNA when run in gel (Lane-1) it also sustained the same configuration of super coiled DNA as in control set. Therefore, the protective nature from *Azolla* may be expected as bio-resource for amelioration of nucleic acid degradation.

In introduction of any cellular mechanism to reduce the loss of DNA in *Azolla* is expected to have improved antioxidant properties. In Figs. 8–11 there are distinct representations where *Azolla* exhibited variations in polymorphism for different proteins recruited for anti-oxidation. In enzymatic antioxidation *Azolla* exhibited two forms of peroxidase varying with electron donors as guaiacol and ascorbate to lyse the H_2O_2. Thus, plants responses to interact with H_2O_2 by GPX and APX activity which are compatible in nature where a dose dependent relationship was the feature. For both the enzymes the activities were linear in manner with peak value of 1.68 and 2.17 folds for GPX and APX respectively compared to control. The significant variations with regards to concentration would be a pathway for plant to reduce

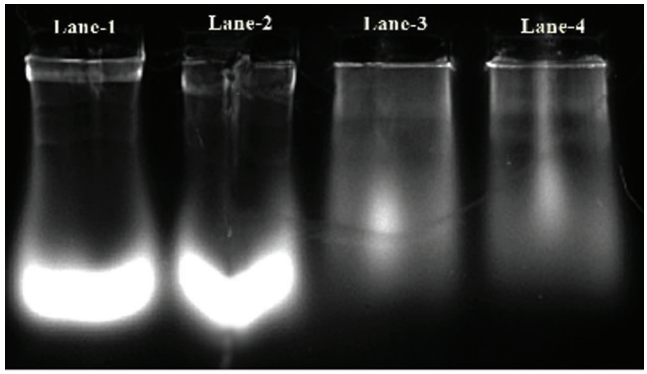

Figure 7: Plasmid nick assay demonstrating fragmentations of DNA released in different lanes (1 to 4) of 1% agarose gel under different treatments as indicated within the panel

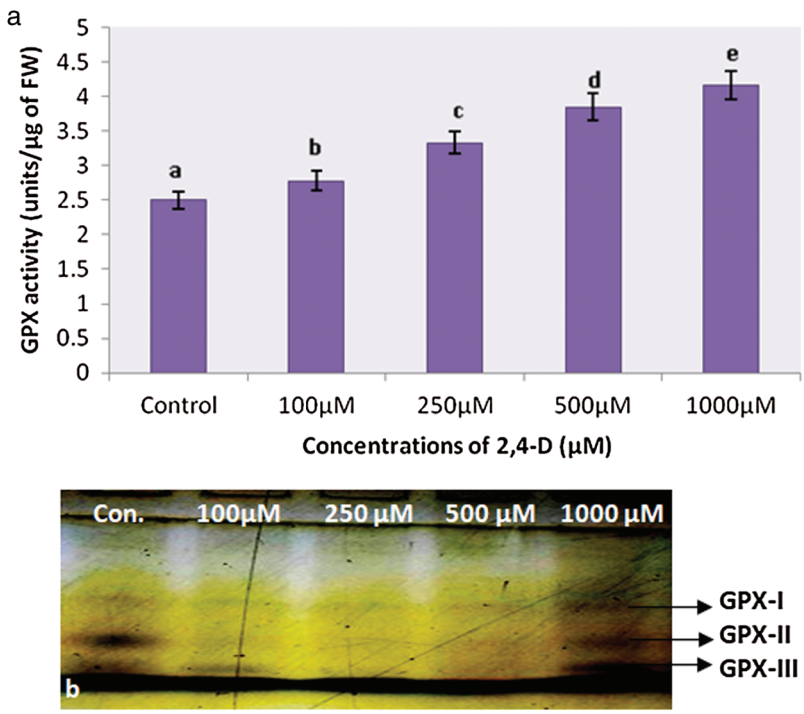

Figure 8: *In-vitro* (a) and *in-gel* (b) activity of guaiacol peroxidase activity under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

the H_2O_2 utilizing both forms of peroxidase. Plants responded more when two peroxidases were produced with distinct variations of polymorphism. For GPX their recorded variable numbers of bands with gradual increase in concentration through 2,4-D treatments. As compared to other antioxidatives enzymes, APX is

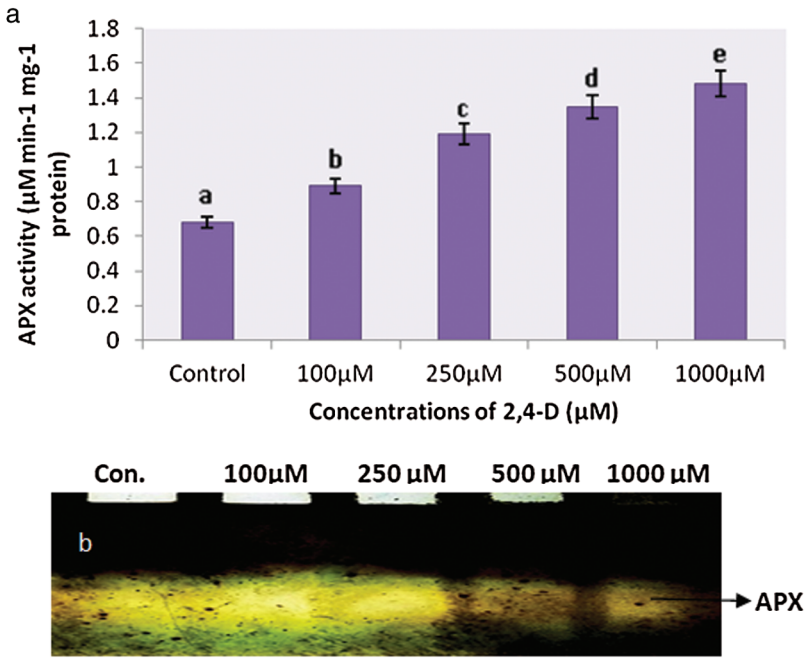

Figure 9: *In-vitro* (a) and *in-gel* (b) activity of ascorbate peroxidase activity under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

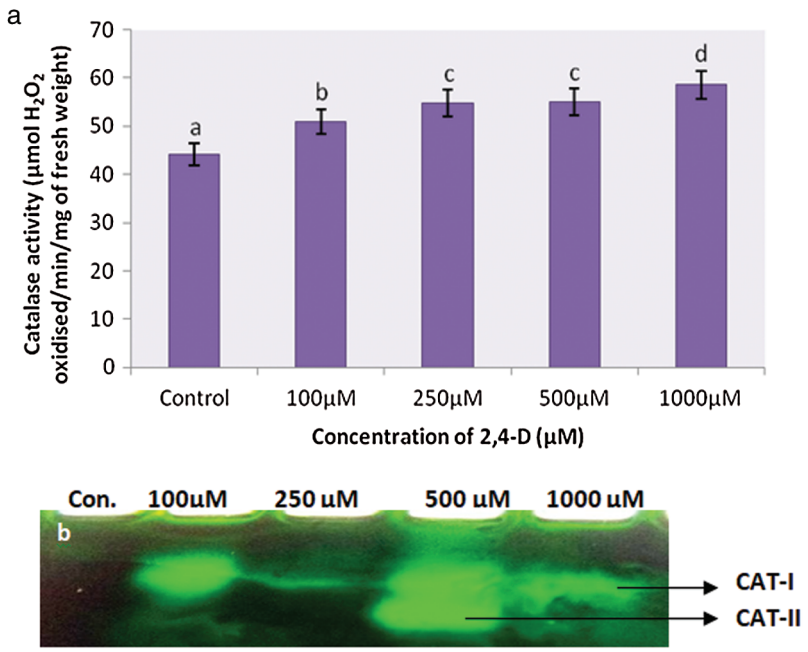

Figure 10: *In-vitro* (a) and *in-gel* (b) activity of catalase activity under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

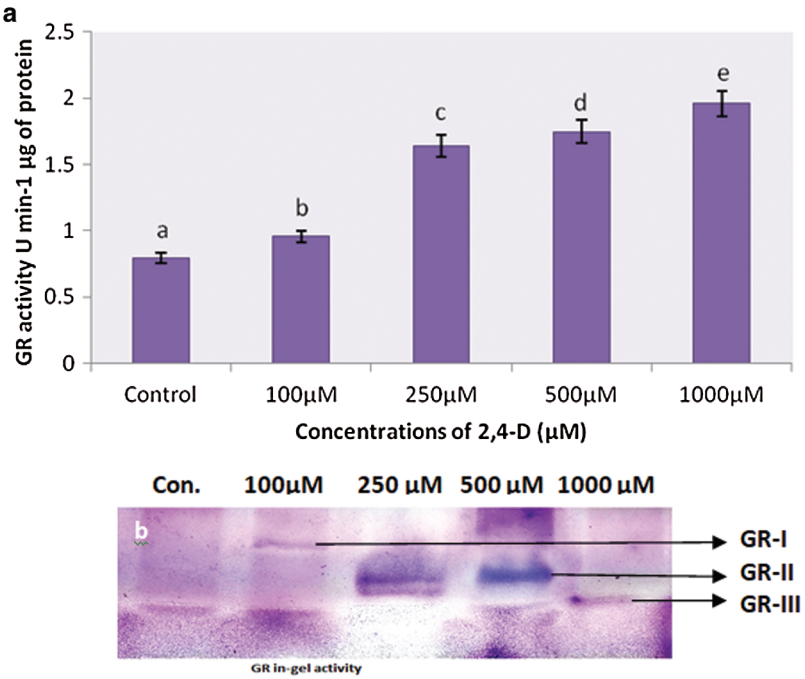

Figure 11: *In-vitro* (a) and *in-gel* (b) activity of glutathione reductase activity under different concentrations 0 µM (Control), 100 µM, 250 µM, 500 µM and 1000 µM of 2,4-D in *Azolla* plants. Bars showing different letters indicate significant differences according to Duncan's test at $p \leq 0.05$

less varied with its polymorphic expression. Another important antioxidative enzyme CAT had more stabilized *in-vitro* activities to reduce the H_2O_2 content as recorded through 2,4-D treatments (Fig. 10a). Interesting to note that initially plant had induced the CAT activity by 1.11 folds and sustained in more stable manner, still, the maximum activity was recorded at 1000 µM which was 1.3 folds over the control. On the basis of expression potential of polymorphic band number and intensity, CAT showed the most discriminatory trend (Fig. 10b). One of those appeared most strikingly concentrated as progressed though the couses of 2,4D when compared to control. Thus, at penultimate concentration of herbicide plants recorded two distinct bands which again reduced to only one when mate with 1000 µM 2,4-D.

In assessment of redox homeostasis, glutathione metabolism was monitored with support of GR activities (Fig. 11). The evidences for plants depletion of reduced glutathione were established with up regulation of GR activity. During *in-vitro* analysis of GR activity, a linear relationship was observed with ongoing concentration of 2,4-D which upregulated by 2.45 folds over the control. Still, at initial concentration activity of GR was not significantly ($p \leq 0.05$) effected contributing glutathione metabolism was not significant. On the other hand, polymorphic expression of GR was also consistent with two distinct bands and those were not dose dependent to the 2,4-D concentration. Still, the penultimate concentration of 2,4-D plant maximized significant variations with their distinct bands which again resolved at the maximum concentration.

4 Discussion

In crop production practices herbicide with different molecular configurations have wider ranges of applications as well as varying tolerance to non-targeted species. Absorptions of herbicides either from pre and post-emergent types, the crops are open to be toxicated in a number of ways, particularly, from residual effects in soil. In earlier reports, few micro and macro-flora had registered their tolerance even

under herbicide enriched soil [32]. *Azolla pinnata* R.Br. has already been cited as a potent tolerant species even with a wider bio-sorption of 2,4-D in solution recorded earlier [33]. Here the present experiment would be a document for insights of tolerance in *Azolla* through some significant cellular responses.

In principle, the soil micro flora was displayed with some alterations of cellular activities and established those towards herbicidal tolerance [34]. *Azolla* would be the first report to show a wider bio-sorption of 2,4-D, however, within our experimental syetem. The bioaccumulation of 2,4-D is coupled with some over-expressed physiological activities. In case of soil micro flora, the identified cellular responses towards the tolerance of herbicide were mainly the use of C and N-source from herbicide residue as bio-degraded [35]. The consumption of the herbicides is assumed to be exclusive source of energy through bio-degradation and that otherwise may support the tolerance to xenobiotics [36]. With this background the most appropriate cellular functions targeting the 2,4-D interference would be the IAA profile in *Azolla* tissues. 2,4-D being a phenoxy derivatives of auxin is ought to be compatible for its cellular functions, either through disintegration or any changes. In the present experiment a significant down regulation of cellular IAA concentration through 2,4-D dosages may be suggestive for any of its inhibition of biosynthesis or hydrolysis. 2,4-D as herbicides in sensitive weed species was reported to have a diminished concentration even below the threshold values [37]. Still, the *Azolla* plants could survive within the suppressed IAA concentration for stipulated period and thus assumed to be insensitive to 2,4-D for its sustenance. In other citation sensitivity of 2,4-D toxicity in soil is followed by driving factors for subdued growth and even mortality of the tissues through IAA metabolism. In our earlier communication the *Azolla* was also evident with a significant variance of IAA metabolism through its enzymatic oxidation [12]. Therein, in IAA-oxidase, particularly, in apoplastic space happens to be a significant biomarker for phyto-toxicity of 2,4-D through its variation of expression. IAA is also implicated in typical plant cellular function for osmotic turgidity through increased membrane permeability. For cell wall elongation, IAA is classically linked to increase the membrane integrity protein as well as increasing more negative water potential through solute concentration. Such activity is also facilitated in plant facing any condition approving dehydration [38]. H^+-ATPase a membrane bound protein establishes an H^+ gradient over the membrane to facilitate more accessibility of ions. Understanding herbicidal toxicity for osmotic stress induction, *Azolla* would be involved in any kind of alteration to stabilize the water relation. Thus, activity of H^+-ATPase may serve a reliable biomarker to score in *Azolla* for better tolerance against 2,4-D induced water stress. H^+-ATPase also reported a fair turn-over of osmotic turgidity as well as ion-concentration against water and salinity stress, mostly in crop species. Still, in ferns like *Azolla* it would be equally implicated as expected. This got more relevance when exercise of activators and inhibitors worked proportionately to command on H^+-ATPase activity *vis-a-vis* to restore the ion concentration.

Therefore, membrane properties with regards to ion transport are relevant to consider against herbicidal stress in plants. When *Azolla* cell wall *is* purified and extracted with suitable solvents, a significant amount of free polyamine was eluted from HPLC chromatogram. Though no consistent trend of each polyamine fractions were noted through concentration gradient of 2,4-D, still, an over-expressed pattern for total polyamine was significant. This showed the recruitment of polyamine and regulation also, for corroborating the stress impulses from 2,4-D toxicity. In earlier studies, abiotic stressors with their various modalities were recorded to contribute either shielding of cell wall or/and reducing the oxidative damages in plants. It is the cationic residues of polyamine backbone that shields the anionic domain of the cell membrane protects the specific ion-induced toxicity as well as ROS mediated lipid peroxidation. *Azolla* herein would be no less to have a prospect in over-expression of the polyamine circumventing the tolerance as executed in other crop species.

In contiguous to oxidative stress, plants are expected to have modulated their cellular redox. 2,4-D toxicity has already been established in few cases to have an extent of elicitation for ROS [10]. This

holds also true in the present case, where in *Azolla* system a significant amount of NOX activity registered the sensitivity of tissue for oxidative damages. Within the apoplastioc speces, NOX can transfer one electron to molecular oxygen and thereby converts into super oxide, the most potent free radical.NOX has its dual modes to serve any plant species within its threshold values of activities. Primarily, its overexpresion leads to alleviation of redox inducing peroxidation of bio molecules [39]. Secondarily, the genic regulation of ROS may evoke the cellular defense to activate antioxidation cascade. In our earlier reports 2,4-D exposure in *Azolla* had not any significant impacts for lipid peroxidation and protein oxidation for those of crop species [13]. Therefore, *Azolla* system would be circumvented in NOX activity, in elicitation of towards basal antioxidation pathways. This may encounter any free radical or its by-products occupied in bio molecule degeneration out of 2,4 D exposure. Moreover, the expression variability in NOX for its isozymic profiles should strengthen the genic regulation, however, diffentially for ROS generation with efficiencies.

Activity of peroxidase with its different molecular forms in terms of electron donor is a significant cellular index for anti-oxidative ROS lysis. The present experiment the GPX was displayed with two forms for guaiacol and ascorbate as chemical moieties to donate electrons in ROS reduction. GPX has been variable isozyme forms in different crop species as displayed with *Azolla* also under 2,4-D stress. Activity of GPX recorded a linear rise through the courses of 2,4-D in a dose dependent manner. This also holds true with the increased intensities of individual polypeptide bands in isozymic profiles suggesting H_2O_2 lysis, may solely be dependent on this peroxidase system.

In plant system GPX and APX are complementary to each other in lysis of H_2O_2, still, variable in cell organelle or tissues [40]. This would also be illustrated in case of *Azolla*, where 2,4-D toxicity and its management is based on neutralization of ROS and may be became the prime one apart from cellular sequestering [13]. Regarding the homeostasis of cellular redox under alleviated ROS concentration definite reactions cascades should exists in plants. Glutathione, a redox residue with its oxidized and reduced molecular forms are the most important cellular responses under stress. 2,4-D undoubtedly being a source of oxidative stress as evident in sensitive species are also justified with *Azolla* also. It is expected that variations of glutathione reductase and it's polymorphism may be affiliated in regulation of the oxidative damages. This also advocates for tolerance of *Azolla* to 2,4-D stress by improvising glutathione metabolism in accompany with existing enzymatic anti oxidation. Both the number of isozymic bands and their discrimination in protein intensities through 2,4-D concentration would be an example. The more variations of GR activities along with expression potential must suffice the reduced glutathione to stabilize depleted redox. Moreover, the patterns of isoforms may set the biomarkers corroborating 2,4-D toxicity even with *Azolla* system. On the other hand, CAT activity may also substantiate the residual H_2O_2 lysis pathway where no requirements of phenolic residues are required as electron donor. CAT activity in crop species represents most discriminating trend according to types and even gradient of stressors. This is predominantly the enzymatic protein inhibition over the critical condition of H_2O_2, however, specifically for CAT [41]. Probability also exist where de-novo synthesis of CAT protein also down regulated beyond a threshold concentration of peroxide like other ROS/free radicals. Accordingly, the rise of activity in *Azolla* didn't appear in a more consistent mode and hardly any significance according to concentration of 2,4-D was recorded. Still, in comparison to control condition, *Azolla* exhibited an alleviation of CAT activities and may support the anti-oxidation by lysis of ROS. On molecular profile the polymorphism was more discriminatory and may not characterize any of its polypeptides to demark the tolerance. The initial and penultimate concentration of 2,4-D is exhibited the most promotive in CAT expression and that may otherwise substantiate non-specific nature for this particular protein. Therefore, the equal probability for conflicting nature of activities through 2,4-D doses may not be supportive as bio indicator of 2,4-D toxicity.

Xenobiotic toxicity is finally executed in terms of hydrolysis of nuclear or genic material preeciding the lysis of nuclear membrane. 2,4-D, undoubtedly a ROS generator have been cited for oxidation of chromatins into nucleotides that on separation gives a characteristic features on gel [42]. Comet assay is such a technical device that scores the lysis of nuclear material on any oxidation exposure to the nuclei of affected tissues. In a dose dependent manner 2,4D seems to be inductive in lysis of the nuclear membrane through ROS mediated nucleophilic reactions as also evident in *Azolla* herein. This also holds that 2,4-D is a real xenobiotic residue that actually becomes detrimental in loss of DNA at cellular level. This is more illustrated when *Azolla* extract were monitored for its efficacy with accumulated 2,4-D for *in-vitro* protection against lysis of nuclear material. Thus, pUC19 base plasmids represented the good material. Thus, pUC19 base plasmids represented the good material in comparison in comparison to other artificial ROS inducer as found in the experiment when compared to *Azolla* extract. This is clear showed *Azolla* species might have induced it's any of the bio-constituents that have adequate ability to restore the nuclear membrane integrity. This is clearly evident when *Azolla* extract was documented to retain the plasmid native form in electrophoresis system as compared to chemical residues (Fenton's reagent) as well as direct application of 2,4-D. This is more enthusiastic to approach the *Azolla* and also like other non-cryptogamic species to explore their potential either to combat the nuclear disintegration or to retrieve degenerated nucleic acid material from stressors like xenobiotics and others [43].

5 Conclusion

The present study aimed to evaluate *Azolla* with its efficacy of 2,4-D tolerance and thereby its possible utilization in biological mitigation for xenobiotic pollution. With the wider spectrum of 2,4-D biosorption following changes in few physiological and cellular activities, this species may be established as a good phyto-remediator. On physiological insights *Azolla* can tolerate 2,4-D toxicity by two means: primarily, adjusting the auxin metabolism by IAA-oxidase and secondarily, by improving the anti-oxidation activity. Catabolism of auxin with IAA-oxidase activity and its possible polymorphisms must be the relevant to screen this species against the herbicide. This is more established when plants recorded an adjustment of membrane properties with H^+-ATPase activities in alteration. Contextually, the variations of polyamine concentrations detected in *Azolla* with respect to 2,4-D toxicity appeared to support the membrane integrity. In amelioration of oxidative damages *Azolla* was also evident with its in-built antioxidation mechanism to quench the ROS. This got evident from restriction of subsequent lysis of DNA as detected through plasmid nick assay as well as confirmed with comet assay. A few possible polymorphisms of different antioxidative proteins have given the impetus to this fern as improved bio-resource for anti-oxidation. Therefore, *Azolla* has significant commemoration as a bio-resource to mitigate the herbicidal stress like xenobiotic toxicity with its improved anti-oxidation pathways. Still, it may be understood that more in-depth insights are required to realize its real value as a xenobiotic hyper accumulator vis-à-vis tolerant plant species. With this a field based trial in natural condition of *Azolla* is needed to revalidate its beneficiaries in xenobiotic phyto-remediation for sustainable agriculture.

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